Inhibitors of Polyamine Biosynthesis VII: Evaluation of Pyruvate Derivatives as Inhibitors of *S*-Adenosyl-L-methionine Decarboxylase

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Abstract □ The mechanism of the enzymatic decarboxylation of *S*-adenosyl-L-methionine catalyzed by *S*-adenosyl-L-methionine decarboxylase and its inhibition by methylglyoxal bis(guanylhydrazone) were investigated. The results indicate that the carbonyl group of the pyruvate cofactor does not form an azomethine bond with an amino group of the enzyme protein. The substrate and/or product forms an azomethine bond with the pyruvate cofactor, which can be reduced efficiently with sodium cyanoborohydride. Methylglyoxal bis(guanylhydrazone) appears to interfere with the formation of the enzyme–substrate complex by competing with the substrate for binding with the active enzyme site. The dimethylaminoethylhydrazone, semicarbazone, and guanylhydrazone derivatives of pyruvic acid, ethyl pyruvate, pyruvic acid amide, and pyruvyl glycineamide were synthesized. None of these compounds had significant inhibitory activity on the enzymatic decarboxylation of *S*-adenosyl-L-methionine by *S*-adenosyl-L-methionine decarboxylase from rat liver *in vitro*. These results indicate that the structural requirements for binding of methylglyoxal bis(guanylhydrazone) to the enzyme are strict and that structural modifications of this compound result in a dramatic loss of activity.

Keyphrases □ *S*-Adenosyl-L-methionine decarboxylase—inhibition by pyruvate derivatives □ Enzyme inhibition—effects of pyruvate derivatives on *S*-adenosyl-L-methionine decarboxylase

The polyamines appear to play an essential role in cellular metabolism and cell proliferation (1). Selective inhibitors of polyamine biosynthesis have produced antiproliferative activity in cell culture (2) and *in vivo* (3). Most of the compounds studied have been inhibitors of L-ornithine decarboxylase, the rate-controlling enzyme in the biosynthesis of the polyamines (4). Only a few inhibitors of *S*-adenosyl-L-methionine decarboxylase, the second enzyme in the synthetic pathway of polyamines, have been studied (5, 6). Therefore, studies to develop specific inhibitors of mammalian, putrescine-activated *S*-adenosyl-L-methionine decarboxylase were initiated.

This report presents a tentative scheme for catalysis by *S*-adenosyl-L-methionine decarboxylase and a possible mechanism of its inhibition by methylglyoxal bis(guanylhydrazone) (I*a*). The synthesis of some pyruvate derivatives structurally similar to I*a* and evaluation of them as inhibitors of enzymatic decarboxylation of *S*-adenosyl-L-methionine by the decarboxylase from rat liver also are reported.

BACKGROUND

The identification of covalently bound pyruvate in rat liver *S*-adenosyl-L-methionine decarboxylase was reported recently in two studies (7, 8). The pyruvate group appears to be essential for the catalytic activity of the enzyme and probably is involved in the formation of an azomethine bond with the amino group of the substrate, *S*-adenosyl-L-methionine, to form an intermediate (IV) (Scheme I). Previous studies with pyridoxal phosphate-dependent decarboxylases showed that the pyridoxal phosphate is linked to the ε-amino group of a specific lysyl residue by an azomethine bond and that the enzyme–substrate complex is formed by transaldimination between the enzyme–pyridoxal phosphate complex and the substrate. Unlike pyridoxal-dependent decarboxylases, the carbonyl group of the pyruvate cofactor does not form an azomethine bond with an amino group from the enzyme protein since sodium borohydride reduction of the purified enzyme results in the formation of lactic acid and not an *N*-substituted alanine.

Since studies with model systems indicated that transaldimination proceeds more rapidly than *de novo* Schiff base formation (9), the possibility of *S*-adenosyl-L-methionine decarboxylase activation by putrescine, due to the formation of the ketimine (III) between the pyruvate residue and putrescine, was explored. Transketimination between III and the substrate (II) would result in the more rapid formation of the intermediate (IV). The decarboxylation product (V) of II also may be bound to the enzyme by an azomethine bond.

Imminium compounds such as III–V could be trapped in the presence of free pyruvate residues by selective reduction with cyanoborohydride (10). Thus, the presence of such intermediates in an incubation medium containing *S*-adenosyl-L-methionine decarboxylase and the appropriate ligand could be demonstrated if a loss of enzymatic activity were observed on treatment of the mixture with sodium cyanoborohydride. This approach was used by Satre and Kennedy (11) to demonstrate the formation of a Schiff base between the amino group of phosphatidyl serine and the pyruvate residue of the enzyme as an essential step in the action of phosphatidylserine decarboxylase. Therefore, experiments were carried out to determine the effects on enzyme activity of the addition of sodium cyanoborohydride to *S*-adenosyl-L-methionine decarboxylase preparations containing the activator putrescine and/or the substrate.

EXPERIMENTAL[1]

Melting points were determined in open capillary tubes and are uncorrected. NMR spectra were taken in deuterochloroform or deuterium oxide with tetramethylsilane or sodium 2,2-dimethyl-2-silapentane-5-sulfonate as the internal standard, respectively. Mass spectral analyses were performed at 20–70 ev and chamber temperatures of 25–200°.

Methylglyoxal Disemicarbazone (I*b*)—Methylglyoxal (2.5 g of a 40% aqueous solution) was added to a solution of semicarbazide hydrochloride (1.0 g) and sodium acetate (1.5 g) in water (25 ml). The mixture was heated on a steam bath for 30 min. The precipitate was collected by filtration, washed with warm water, and dried *in vacuo* to give I*b* (2.4 g, 95% yield), mp 252° dec. [lit. (12) mp 254°].

Anal.—Calc. for $C_5H_{10}N_6O_2$: C, 32.25; H, 5.41; N, 45.14. Found: C, 32.47; H, 5.43; N, 45.10.

Methylglyoxal Dithiosemicarbazone (I*c*)—A solution of sodium acetate (2.0 g in 10 ml of water) was added to a solution of methylglyoxal (2.5 g of a 40% aqueous solution) and thiosemicarbazide (1.0 g) in water (25 ml). The mixture was heated on a steam bath for 30 min. A heavy orange precipitate was formed and then collected by filtration. The precipitate was washed with boiling water and dried *in vacuo* overnight to give Ic (2.8 g, 93% yield), mp 250° dec. [lit. (12) mp 253°].

Anal.—Calc. for $C_5H_{10}N_6S_2$: C, 27.51; H, 4.62; N, 38.50. Found: C, 27.77; H, 4.46; N, 38.47.

Pyruvyl Glycineamide Diethyl Ketal (XI)—Ethyl pyruvate diethyl ketal was prepared from ethyl pyruvate and ethyl orthoformate according to the procedure of Wermuth and Marx (13). Ethyl pyruvate diethyl ketal (5.0 g, 26 mmoles) was treated with a 3% aqueous potassium hydroxide solution (50 ml, 30 mmoles) and stirred overnight at room temperature. The aqueous solution was made acidic by the addition of 5% HCl and extracted with ether (2 × 150 ml). The ether extract was concentrated *in vacuo* to give pyruvic acid diethyl ketal (3.5 g, 88% yield); IR (neat):

[1] Instruments used were a Thomas-Hoover melting-point apparatus, a Perkin-Elmer 281 spectrophotometer, Varian A-60D and T-60 NMR spectrophotometers, an AEI MS-30 mass spectrometer, and a Beckman LS-100C liquid scintillation counter. Elemental analyses were performed by M-H-W Laboratories, Phoenix, Ariz.

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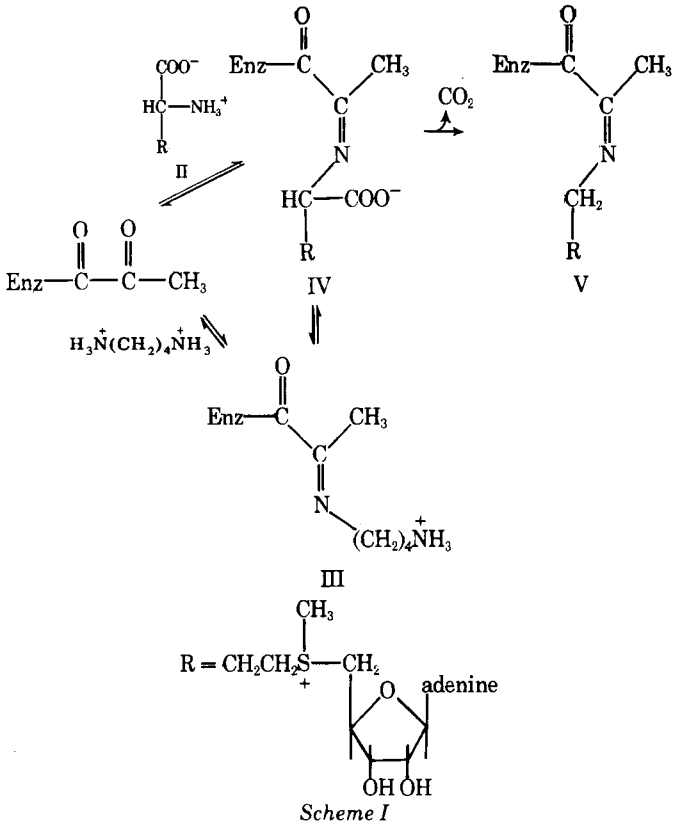


Scheme I

3200 (broad), 2985, 2930, 2895, 1740, 1450, 1370, 1150, 1040, 950, 855, and 740 cm^{-1}; NMR (deuterochloroform): δ 3.6 (m, 4H), 1.6 (s, 3H), and 1.2 (t, 6H) ppm.

A cooled (ice–salt bath) solution of pyruvic acid diethyl ketal (2.0 g, 12 mmoles) in chloroform (100 ml) was treated with triethylamine (1.25 g, 12 mmoles) and ethyl chloroformate (1.35 g, 12 mmoles), and the mixture was stirred for 20 min. The ice–salt bath was removed, and a solution of glycineamide (1.0 g, 15 mmoles) in chloroform (100 ml) was added slowly over 30 min. The reaction mixture was stirred for 4 hr at room temperature, and the chloroform solution was washed with successive portions of 10% $NaHCO_3$ and brine. The chloroform solution was dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo*. The semisolid residue obtained was crystallized from boiling ether to give XI (1.6 g, 60% yield), mp 95–96°; IR (melt): 3320, 2995, 2940, 1670, 1620, 1525, 1450, 1365, 1300, 1200, 1160, 1050, and 950 cm^{-1}; NMR (deuterochloroform): δ 7.6, 6.9, and 6.2 (broad s, 3H, exchangeable with deuterium oxide), 4.0 (d, 2H), 3.5 (q, 4H), 1.5 (s, 3H), and 1.3 (t, 6H) ppm; mass spectrum: *m/e* 219 ($M^+ + 1$), 217 ($M^+ - 1$), 173, 156, 128, 117, 100, 89, 75, 61 (100%), 59, 43, and 30.

Anal.—Calc. for $C_9H_{18}N_2O_4$: C, 49.53; H, 8.31; N, 12.84. Found: C, 49.25; H, 8.49; N, 12.89.

Monoacetyl Hydrazine—Monoacetyl hydrazine was prepared according to the procedure described by Rabini and Vita (14), mp 64–65° [lit. (14) mp 62°]. This compound is hygroscopic and was stored in a vacuum desiccator over anhydrous calcium chloride granules.

***N*-Acetyl-*N*-(dimethylaminoethyl)hydrazine Dihydrochloride (XIV)**—A solution of dimethylaminoacetaldehyde diethyl acetal (XII) (5.0 g, 31 mmoles) in concentrated hydrochloric acid (10 ml) was allowed to stand overnight at 4° and then was concentrated under reduced pressure at 40°. The viscous residue was dissolved in dry methanol (100 ml) and treated with acetylhydrazine (2.5 g, 35 mmoles) and sodium cyanoborohydride (1.3 g, 20 mmoles) under nitrogen. The pH of the reaction mixture was maintained at 2–3 by the addition of methanolic hydrochloric acid, using bromcresol green as an indicator, over 6 hr at room temperature.

After concentration *in vacuo* at room temperature, the residue was dissolved in cold absolute ethanol and filtered. The filtrate was treated with ethanolic hydrochloric acid and stored at 4° overnight. The precipitated solid was filtered and dried *in vacuo* to give XIV (2.8 g, 42% yield), mp 126–128°. An analytical sample was recrystallized from 95% ethanol; IR (KBr): 3400 (broad), 300 (broad), 2040, 1715, 1630, 1460, 1380, 1230, 1000, and 800 cm^{-1}; NMR (deuterium oxide): δ 3.2 (s, 4H), 2.8 (s, 6H), and 2.0 (s, 3H) ppm; mass spectrum: *m/e* 145 ($M^+ - 2HCl$), 87, 71, 59, 58 (100%), 44, and 36.

Anal.—Calc. for $C_6H_{17}Cl_2N_3O$: C, 33.03; H, 7.86; N, 19.26. Found: C, 33.17; H, 8.07; N, 19.20.

Dimethylaminoethylhydrazine Dihydrochloride (XV)—A solution of XIV (2.0 g, 9 mmoles) was dissolved in ethanolic hydrogen chloride solution (20 ml) and warmed gently on a steam bath for 45 min. The mixture was stored at 4° overnight to give a quantitative yield of XV, mp 120–121°. The product was hygroscopic; IR (KBr): 3400 (broad), 3000, 2700, 2050, 1620, 1470, 1150, and 800 cm^{-1}; NMR (deuterium oxide): δ 3.2 (s, 4H) and 2.8 (s, 6H) ppm; mass spectrum: *m/e* 103 ($M^+ - 2HCl$), 58 (100%), 42, and 36.

Anal.—Calc. for $C_4H_{15}Cl_2N_3$: C, 27.28; H, 8.59; N, 23.86. Found: C, 27.47; H, 8.56; N, 23.96.

Dimethylaminoethylhydrazone Derivatives (VI*a*–VI*d*)—The ketone (IX*a*, IX*b*, or IX*c*) or XI (3 mmoles) was added to a solution of XV (0.5 g, 3 mmoles) in ethanol (20 ml) and allowed to stand overnight at room temperature. Ether was added dropwise to the clear solution until turbidity was observed. The mixture was stored at 4°. The precipitated solid was filtered and crystallized to give the desired products (VI*a*–VI*d*) (Table I).

Semicarbazone Derivatives (VII*a*–VII*d*)—One gram of the ketone (IX*a*, IX*b*, or IX*c*) or XI was added to a solution of semicarbazide hydrochloride (1.0 g) and sodium acetate (1.5 g) in water (25 ml). The mixture was heated on a steam bath for 15 min and allowed to stand overnight. The formed solids were filtered, recrystallized from water, and dried *in vacuo* for 12 hr to give the desired products (VII*a*–VII*d*) (Table I).

Guanylhydrazone Derivatives (VIII*a*–VIII*c*)—A suspension of aminoguanidine bicarbonate (50 mmoles) in water (50 ml) was adjusted to pH 5–6 with concentrated hydrochloric acid. Compound IX*a*, IX*b*, or IX*c* was added dropwise to the clear solution of the aminoguanidine, and the reaction mixture was stirred overnight at room temperature. The precipitated solids were filtered, crystallized, and dried overnight *in vacuo* to give the desired products (VIII*a*–VIII*c*) (Table I).

Pyruvyl Glycineamide Guanylhydrazone Hydrochloride (VIII*d*)—Aminoguanidine hydrochloride (0.25 g, 2.5 mmoles) was added to a solution of XI (0.44 g, 2 mmoles) in water (30 ml), and the mixture was heated on a steam bath for 2 hr and stored at 4° overnight. The precipitated crystalline solid was filtered and washed with ice-cold water to give VIII*d* (0.41 g, 87% yield) (Table I).

Pyruvic Acid Guanylhydrazide Guanylhydrazone Dihydrochloride (VIII*e*)—Aminoguanidine hydrochloride (6.6 g, 60 mmoles) was added to a solution of ethyl pyruvate (3.5 g, 30 mmoles) in water (25 ml), and the mixture was heated at 80° overnight. The solution was evaporated to dryness, and the residue after two crystallizations from aqueous ethanol gave VIII*e* (7.3 g, 90% yield), mp 250–251°; IR (KBr): 3200, 2950, 1670, 1590, 1490, 1440, 1420, 1370, 1300, 1150, 1120, 900, and 720 cm^{-1}; NMR (deuterium oxide): δ 1.6 (s, 3H) ppm; mass spectrum: *m/e* 182, 126, 57, 44, 43, 42, 38, and 36 (100%).

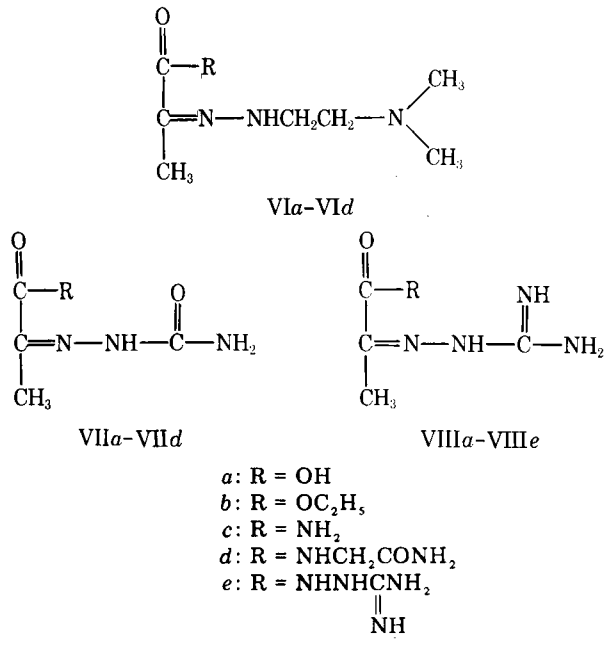


Table I—Analytical Data and Enzyme Inhibitory Activities of Pyruvate Derivatives

Compound	Formula	Yield %	Melting Point	Crystallization Solvent[a]	Inhibition[b], % 20 μM	Inhibition[b], % 10 μM	Analysis, %	Calc.	Found
VI*a*	$C_7H_{15}N_3O_2$·HCl	69	169–170°	A	9	0	C	40.10	40.12
							H	7.69	7.69
							N	20.04	20.20
VI*b*	$C_9H_{19}N_3O_2$·HCl	50	104–105°	B	0	0	C	45.47	45.78
							H	8.48	8.58
							N	17.68	17.86
VI*c*	$C_7H_{16}N_4O$·HCl	78	152–153°	B	2	0	C	40.28	40.64
							H	8.21	8.31
							N	26.85	27.16
VI*d*	$C_9H_{19}N_5O_2$·HCl	70	190–192°	A	3	0	C	40.68	40.40
							H	7.59	7.81
							N	26.36	26.12
VII*a*	$C_4H_7N_3O_3$	95	215–216°	C	0	0	—[c]		
VII*b*	$C_6H_{11}N_3O_3$	93	213–214°	C	8	2	—[c]		
VII*c*	$C_4H_8N_4O_2$	90	241–242°	C	0	0	—[c]		
VII*d*	$C_6H_{11}N_5O_3$	80	240–241°	C	0	0	C	35.81	35.79
							H	5.51	5.43
							N	34.81	34.50
VIII*a*	$C_4H_8N_4O_2$·HCl	87	230–231°	C	2	0	C	26.60	26.93
							H	5.12	5.16
							N	31.03	31.27
VIII*b*	$C_6H_{12}N_4O_2$·HCl	90	150–151°	A	3	0	C	34.54	34.10
							H	6.30	6.16
							N	26.85	26.69
VIII*c*	$C_4H_9N_5O$·HCl	85	255–256°	C	0	0	C	26.75	26.69
							H	5.61	5.65
							N	40.00	39.82
VIII*d*	$C_6H_{12}N_6O_2$·HCl	87	260° dec.	A	0	0	C	28.29	28.61
							H	5.93	5.89
							N	33.01	33.21
VIII*e*	$C_5H_{12}N_8O_2$·HCl	90	250–251°	D	0	0	C	21.99	21.73
							H	5.17	5.00
							N	41.03	40.79

[a] Solvents used were ethanol (A), ethanol–ether (B), water (C), and ethanol–water (D). [b] Inhibition of enzymatic decarboxylation of [1-^{14}C]-*S*-adenosyl-L-methionine by partially purified *S*-adenosyl-L-methionine decarboxylase from rat liver. Compound I*a* produced 100 and 63% inhibition at 10 and 1 μ*M*, respectively. [c] See Ref. 12.

Anal.—Calc. for $C_5H_{12}N_8O$·2HCl: C, 21.99; H, 5.17; N, 41.03. Found: C, 21.73; H, 5.00; N, 40.79.

Enzyme Purification—Rat liver *S*-adenosyl-L-methionine decarboxylase was purified by the method of Pegg (15) with slight modifications. Twelve male Sprague–Dawley[2] rats (250 g) were injected intraperitoneally with methylglyoxal bis(guanylhydrazone)[3] (I*a*, 80 mg/kg) and were sacrificed by cervical dislocation 24 hr later. The livers were removed and homogenized in 200 ml of Buffer A [containing 10 m*M* tris(hydroxymethyl)aminomethane hydrochloride (pH 7.5), 2.5 m*M* putrescine, 1 m*M* dithiothreitol, and 0.1 m*M* ethylenediaminetetraacetic acid disodium] at 4°. All subsequent operations were carried out at 0–5°.

The homogenate was centrifuged at 105,000×*g* for 90 min. The supernate was treated with ammonium sulfate, and the protein fraction precipitating between 35 and 65% saturation was collected by centrifugation. The protein pellet was dissolved in the minimum amount of Buffer A, and the solution was dialyzed overnight against 4 liters of the same buffer.

A glass column (2.5 × 30 cm) was packed with Sepharose[4] linked to I*a* and washed with 200 ml of Buffer A. The dialyzed protein sample was applied to the column at a flow rate of 35–40 ml/hr, and the column was washed with 100 ml of Buffer A followed by 150 ml of Buffer A containing 0.3 *N* NaCl. The protein having *S*-adenosyl-L-methionine decarboxylase activity was eluted off the column with Buffer A containing 0.3 *N* NaCl and 1 m*M* I*a*. The column eluates were collected in 10-ml fractions, and each fraction was assayed for decarboxylase activity using 50 μl in the standard procedure.

Decarboxylase activity usually was found in fractions 4–6, with >80% of the recovered activity in fraction 5 alone. The presence of I*a* in the elution solvent greatly reduced the apparent activity of these fractions. Fractions containing decarboxylase activity were pooled, divided into 5-ml aliquots, and stored at 0–5°. The protein solution was dialyzed against two 1-liter portions of Buffer A, to remove the inhibitor (I*a*), immediately before use as a source of the decarboxylase enzyme. By using this method, an enzyme preparation having a specific activity of ~15 nmoles of carbon dioxide/min/mg of protein routinely was obtained.

Assay of *S*-Adenosyl-L-methionine Decarboxylase Activity—*S*-Adenosyl-L-methionine decarboxylase activity was determined by a modification of a literature method (16). The assay medium contained 0.1 *M* sodium phosphate buffer (pH 7.0), 2.5 m*M* dithiothreitol, 2.5 m*M* putrescine, 0.2 m*M* [1-^{14}C]-*S*-adenosyl-L-methionine (2.0 × 10^5 cpm), and the enzyme fraction[5] in a total volume of 1.0 ml. Reactions were carried out in 16 × 100-mm test tubes sealed with rubber stoppers carrying a polypropylene well[6] containing 0.3 ml of ethanolamine.

After incubation for 30 min at 37°, the reaction was terminated and [^{14}C]carbon dioxide was released by the injection of 0.5 ml of 5 *N* H_2SO_4 through the rubber stopper. The tubes were incubated for an additional 45 min at 25°, and the well and its contents were transferred into a glass scintillation vial containing 10 ml of scintillation cocktail[7]. The samples were stored in the dark overnight and then counted. All counts were corrected for the nonenzymatic production of [^{14}C]carbon dioxide, which was determined by replacing the enzyme fraction in the incubation medium with an equal volume of Buffer A. This method gave the quantitative recovery of the [^{14}C]carbon dioxide released from sodium [^{14}C]-bicarbonate.

Enzyme Inhibition—The test compounds were dissolved in water (I*a* and VI–VIII) or 5% dimethyl sulfoxide in water (I*b* and I*c*) to give 1 m*M* solutions. The incubation medium contained 0.1 *M* sodium phosphate buffer (pH 7.0), 2.5 m*M* dithiothreitol, 2.5 m*M* putrescine, 0.2 m*M* [1-^{14}C]-*S*-adenosyl-L-methionine (2.0 × 10^5 cpm), the inhibitor at concentrations of 50, 20, 10, and 1 μ*M*, and 0.1 ml of the enzyme preparation in a total volume of 1.0 ml. The amount of [^{14}C]carbon dioxide released was measured as described for the assay of *S*-adenosyl-L-methionine decarboxylase activity and corrected for the nonenzymatic release of [^{14}C]carbon dioxide. The presence of dimethyl sulfoxide in solutions of I*b* and I*c* resulted in <5% inhibition of enzymatic activity, and the final

[2] Bio-Lab Corp., St. Paul, Minn.
[3] Aldrich Chemical Co., Milwaukee, Wis.
[4] Prepared according to the procedure described in Ref. 15; Sigma Chemical Co., St. Louis, Mo.
[5] Enzyme fractions were column eluates after recovery of the protein from the Sepharose column or dialyzed protein after preincubation (Tables II and III).
[6] Kontes Glass Co., Vineland, N.J.
[7] Aquasol 2, New England Nuclear, Boston, Mass.

Table II—Effects of pH and Buffer on the Recovery of *S*-Adenosyl-L-methionine Decarboxylase Activity

Buffer	Activity Remaining[a] pH 6.5	pH 7.0	pH 7.5
Tris(hydroxymethyl)aminomethane hydrochloride (10 m*M*)	69	83	58
Phosphate (100 m*M*)	86	100	75

[a] See *Experimental.*

results for these compounds were corrected for inhibition by the solvent.

Effects of pH and Buffer on Recovery of *S*-Adenosyl-L-methionine Decarboxylase Activity—The dialyzed protein fraction containing *S*-adenosyl-L-methionine decarboxylase activity was diluted with Buffer A to a concentration of 0.2 mg of protein/ml. Aliquots (0.1 ml) were diluted to 0.4 ml with one of the buffer solutions listed in Table II and incubated at 37° for 30 min. Aliquots (0.1 ml) were removed and assayed for *S*-adenosyl-L-methionine decarboxylase activity as described for the assay of *S*-adenosyl-L-methionine decarboxylase activity. The activity of the sample incubated in pH 7.0 phosphate buffer was arbitrarily assigned a value of 100, and the activities of the other samples are reported in Table II as a percentage of that activity. Incubation in pH 7.0 phosphate buffer resulted in a 10% loss of activity of the original preparation.

Effects of Different Preincubation Conditions on Recovery of *S*-Adenosyl-L-methionine Decarboxylase Activity—Protein fractions containing *S*-adenosyl-L-methionine decarboxylase activity were dialyzed against a pH 7.5 buffer containing 10 m*M* tris(hydroxymethyl)aminomethane hydrochloride, 1 m*M* dithiothreitol, and 0.1 m*M* ethylenediaminetetraacetic acid disodium. Aliquots of the dialyzed protein were diluted with buffer solution (pH 7.0) containing the additions described in Table III and incubated at 37° for 30 min.

Each sample was dialyzed against Buffer A (two 100-ml portions), and 0.1-ml aliquots of the dialyzed protein were assayed for *S*-adenosyl-L-methionine decarboxylase activity as described for the assay of *S*-adenosyl-L-methionine decarboxylase activity. The activity of the sample preincubated in the buffer with no additions was arbitrarily assigned a value of 100, and the activities of the other samples are reported in Table III as a percentage of that activity.

RESULTS

Incubation of partially purified *S*-adenosyl-L-methionine decarboxylase from rat liver at 37° in a buffer for 30 min resulted in a slight loss of activity. This result is consistent with the previously reported instability of the enzyme at elevated temperatures (16). The nature and pH of the buffer influenced the degree of activity loss (Table II), with optimum stability of the enzyme observed in pH 7.0 phosphate buffer. Addition of sodium cyanoborohydride at 5 and 50 m*M* to the incubation medium [pH 7.0 tris(hydroxymethyl)aminomethane hydrochloride buffer] did not cause additional loss of enzymatic activity (Table III).

Incubation of *S*-adenosyl-L-methionine decarboxylase in pH 7.0 tris(hydroxymethyl)aminomethane hydrochloride buffer containing various concentrations of *S*-adenosyl-L-methionine at 37° for 30 min, followed by dialysis, resulted in a loss of enzymatic activity in the recovered protein. This loss was dependent on the concentration of II in the initial incubation mixture (Table III). Addition of Ia at 1.0 μM to the original incubation mixture resulted in protection of the enzyme from the substrate-induced deactivation.

Addition of sodium cyanoborohydride at 5 and 50 m*M* to a mixture of the decarboxylase and II (0.2 m*M*) in pH 7.0 tris(hydroxymethyl)-aminomethane hydrochloride buffer, followed by incubation at 37° for 30 min, resulted in an essentially complete loss of enzymatic activity in the dialyzed protein. The use of [*S*-C^{3}H$_3$]II (10.13 Ci/mmole) in the experiment resulted in the incorporation of radioactivity in the protein fraction. Extensive dialysis of the protein did not dissociate the radioactivity from the enzyme. The addition of Ia at 0.1 μM significantly lowered the degree of inactivation of *S*-adenosyl-L-methionine by sodium cyanoborohydride treatment in the presence of the substrate and putrescine (Table III).

Addition of sodium cyanoborohydride to a mixture of *S*-adenosyl-L-methionine decarboxylase and putrescine (2.5 m*M*) in pH 7.0 tris(hydroxymethyl)aminomethane hydrochloride buffer and incubation at 37° for 30 min did not result in the loss of enzymatic activity of the dialyzed protein (Table III). The use of [^{3}H]putrescine in the experiment did not lead to the incorporation of radioactivity in the enzyme.

Table III—Effects of Different Preincubation Conditions on the Recovery of *S*-Adenosyl-L-methionine Decarboxylase Activity

Additions to Incubation Medium	Activity Remaining: No Sodium Cyanoborohydride	5 m*M* Sodium Cyanoborohydride	50 m*M* Sodium Cyanoborohydride
None	100[a]	97	96
2.5 m*M* Putrescine	102	101	104
2.5 m*M* Putrescine and 0.2 m*M* *S*-adenosyl-L-methionine	75	4	2
2.5 m*M* Putrescine and 0.1 m*M* *S*-adenosyl-L-methionine	82	ND[b]	ND
2.5 m*M* Putrescine and 0.05 m*M* *S*-adenosyl-L-methionine	90	ND	ND
2.5 m*M* Putrescine, 0.2 m*M* *S*-adenosyl-L-methionine, and 1.0 μM Ia	90	43	34

[a] See *Experimental.* [b] Not determined.

DISCUSSION

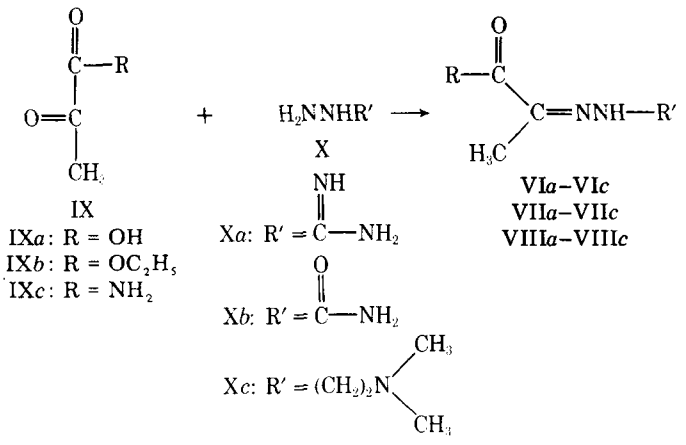
The described experiments confirm that the carbonyl group of the pyruvate cofactor does not form an azomethine bond with an amino group from the enzyme since sodium cyanoborohydride reduction of the enzyme did not result in loss of catalytic activity of the protein. It appears that putrescine does not form the intermediate III and that the mechanism of activation of *S*-adenosyl-L-methionine decarboxylase by putrescine does not involve transketimination between III and the substrate as was proposed initially. However, these experiments indicate that intermediates IV and/or V are formed during the decarboxylation of II by the enzyme.

Methylglyoxal bis(guanylhydrazone) (Ia) appears to interfere with the formation of the enzyme–substrate complex as shown by two experiments: (*a*) Ia protected the enzyme from substrate-induced inactivation, and (*b*) the degree of inactivation of the enzyme by sodium cyanoborohydride in the presence of II was much lower when Ia was added to the incubation medium. These findings and the observation that inhibition of *S*-adenosyl-L-methionine decarboxylase by I may have been competitive with respect to II indicate that Ia competes with II for binding at the enzyme active site.

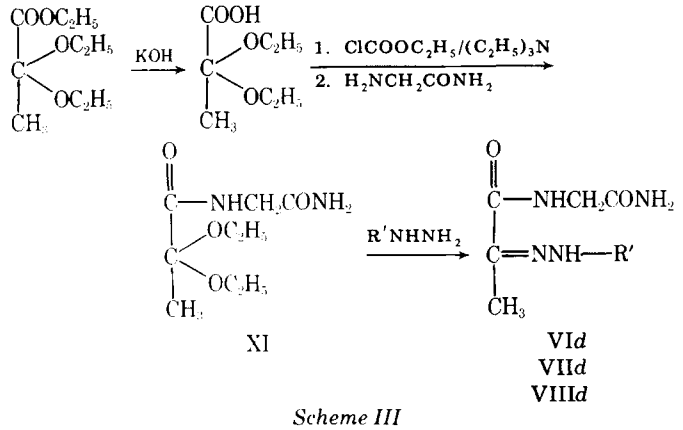
The demonstration that IV and/or V are intermediates in the enzymatic decarboxylation of II prompted the synthesis of some derivatives of pyruvic acid (VI–VIII) and their evaluation as inhibitors of *S*-adenosylmethionine decarboxylase. The structural similarities between VI–VIII and intermediates IV and V suggested that these compounds may form complexes with the enzyme and act as inhibitors of the reaction.

Two other factors suggested that VI–VIII may be inhibitors of *S*-adenosylmethionine decarboxylase. First, Ia, which has structural similarities to intermediates IV and V, inhibits *S*-adenosylmethionine decarboxylase by interfering with the formation of the enzyme–substrate complex. The very high inhibitory activity of Ia (Ia produced 100% inhibition at 10 μM in the presence of 0.2 m*M* substrate) suggested that it may be a transition-state analog rather than an analog of the substrate or product. Second, a number of pyridoxal phosphate analogs, which are structurally similar to the intermediate formed between the cofactor and the substrate, were potent inhibitors of pyridoxal-dependent decarboxylases. However, the intermediates formed between pyridoxal phosphate and substrates are not bound covalently to the enzyme, unlike intermediates IV and V, which would be bound covalently to the enzyme.

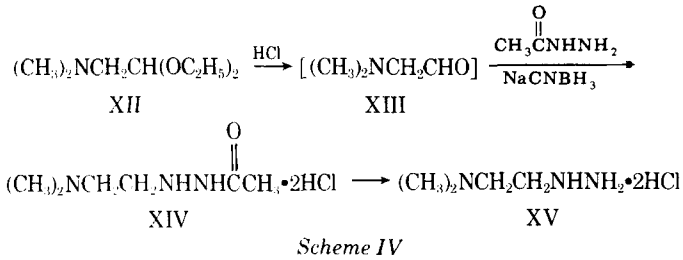
Chemistry—The target compounds (VI–VIII) were synthesized using a modification of the procedure of Podrebarac *et al.* (17) by condensing the appropriate pyruvate derivative with the corresponding hydrazine salt in aqueous alcohol (Scheme II). Pyruvyl glycineamide diethyl ketal (XI) was obtained by the condensation of pyruvic acid diethyl ketal with glycineamide using the mixed carbonic–carboxylic anhydride method (18). Condensation of XI with the appropriate hydrazine provided VId, VIId, and VIIId (Scheme III).



Scheme II

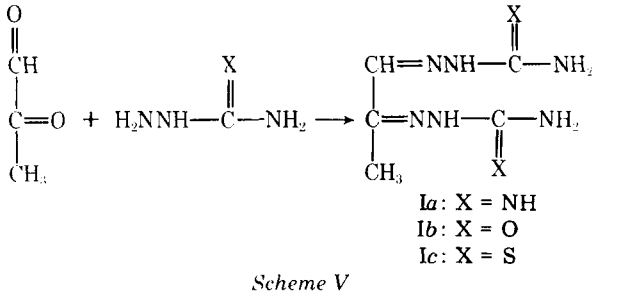


Scheme III



Scheme IV

Acid hydrolysis of dimethylaminoacetaldehyde diethyl ketal (XII) provided the free aldehyde (XIII). Treatment of XIII with acetylhydrazine and sodium cyanoborohydride provided XIV. Acid hydrolysis of XIV provided the dihydrochloride salt, dimethylaminoethylhydrazine dihydrochloride (XV) (Scheme IV). Compounds Ib and Ic were obtained by condensing methylglyoxal pyruvaldehyde with either semicarbazide or thiosemicarbazide in aqueous alcohol using the method of Shriner *et al.* (19) (Scheme V).



Scheme V

Enzyme Inhibition—The inhibition by the test compounds of the enzymatic decarboxylation of *S*-adenosyl-L-methionine was measured *in vitro*. A partially purified protein from rat liver was used as the source of the *S*-adenosyl-L-methionine decarboxylase, and the decarboxylase activity was measured by determining the amount of [^{14}C]carbon dioxide released from *S*-adenosyl-L-methionine labeled with carbon 14 on the carboxyl group. Under the assay conditions, *S*-adenosyl-L-methionine decarboxylase from rat liver had an apparent K_m value for *S*-adenosyl-L-methionine of 5.14×10^{-5} *M* and a V_{max} value of 8.72 nmoles of carbon dioxide/min/mg of protein.

The addition of Ia at 10 μM caused 100% inhibition of the enzymatic activity. However, the addition of Ib or Ic at 100 μM resulted only in 5 and 3% inhibition of enzymatic activity, respectively. Compounds VI–VIII showed little or no inhibitory activity when added to the reaction mixture in concentrations of 10 and 20 μM (Table I).

These results indicate that the structural requirements for binding of Ia to the enzyme are strict and that structural modifications of this compound result in a dramatic loss of activity. The low inhibitory activities of Ib and Ic underscore the essential role the strongly basic guanidino groups play in the binding of Ia to the enzyme. The lack of significant inhibitory activities for VI–VIII indicates that the inhibitory activity of Ia is not due to its structural similarity to intermediates IV and V in the enzymatic reaction.

REFERENCES

(1) J. Janne, H. Poso, and A. Raina, *Biochim. Biophys. Acta*, **473**, 241 (1978).

(2) P. S. Mamont, M. C. Duchesne, J. Grove, and P. Bey, *Biochem. Biophys. Res. Commun.*, **81**, 58 (1978).

(3) N. J. Parkash, P. J. Schechter, J. Grove, and J. Koch-Weser, *Cancer Res.*, **38**, 3059 (1978).

(4) M. M. Abdel-Monem and E. A. Mikhail, *J. Pharm. Sci.*, **67**, 1174 (1978), and references cited therein.

(5) A. Corti, C. Dave, H. G. Williams-Ashman, E. Mihich, and A. Schenone, *Biochem. J.*, **139**, 351 (1974).

(6) A. E. Pegg, *J. Biol. Chem.*, **253**, 539 (1978).

(7) A. E. Pegg, *FEBS Lett.*, **84**, 33 (1977).

(8) A. A. Demetriou, M. S. Cohen, C. W. Tabor, and H. Tabor, *J. Biol. Chem.*, **253**, 1684 (1978).

(9) E. H. Cordes and W. P. Jencks, *Biochemistry*, **1**, 773 (1962).

(10) R. F. Borch, M. D. Bernstein, and H. D. Durst, *J. Am. Chem. Soc.*, **93**, 2897 (1971).

(11) M. Satre and E. P. Kennedy, *J. Biol. Chem.*, **253**, 479 (1978).

(12) "CRC Handbook of Tables for Organic Compound Identification" 3rd ed., R. C. Weast, Ed., Chemical Rubber Co., Cleveland, Ohio, 1967.

(13) C.-G. Wermuth and H. Marx, *Bull. Soc. Chem. Fr.*, **1964**, 732.

(14) T. Rabini and G. Vita, *J. Org. Chem.*, **30**, 2486 (1965).

(15) A. Pegg, *Biochem. J.*, **141**, 581 (1974).

(16) A. E. Pegg and H. G. Williams-Ashman, *J. Biol. Chem.*, **244**, 682 (1969).

(17) E. G. Podrebarac, W. H. Nyberg, F. A. French, and C. C. Cheng, *J. Med. Chem.*, **6**, 283 (1963).

(18) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Wiley, New York, N.Y., 1961, p. 978.

(19) R. L. Shriner, R. C. Fuson, and D. Y. Curtin, "The Systemic Identification of Organic Compounds," Wiley, New York, N.Y., 1964.

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